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(54) Title: INHIBITION OF CELL PROLIFERATION BY E2F-1 ANTISENSE OLIGONUCLEOTIDES

(57) Abstract

Antisense oligonucleotides specific for the E2F-1 gene inhibit proliferation of cells. The oligonucleotides are useful in the treatment of neoplastic diseases, primarily neoplastic diseases characterized by the growth of solid tumors. The E2F-1 antisense oligonucleotides are in particular believed useful for treating those cancers characterized by the inactivation of functional retinoblastoma tumor surpressor gene expression.

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INHIBITION OF CELL PROLIFERATION BY E2F-1 ANTISENSE OLIGONUCLEOTIDES

Field of the Invention

The invention relates to antisense oligonucleotides, in particular to antisense oligonucleotides to the nuclear protein E2F-1 gene, and the use of such oligonucleotides to inhibit proliferation of cells.

Reference to Government Grant

The invention described herein was supported in part by National Institutes of Health grant CA46782. The United States Government has certain rights in the invention.

Background of the Invention

During the G_1 phase of the cell cycle a series of factors involved in the transition to S phase are coordinately activated. Genes expressed in mid-late G_1 such as c-myb, cdc2, cyclin D1 and DNA polymerase-α, are turned on before the onset of DNA synthesis and are required for cell cycle progression, DNA synthesis and mitosis. The transcription factor E2F was initially defined as a cellular factor required for the activation of the adenovirus E2 promotor (Yee et al., EMBO J. 6: 2061-2068, (1987)).

Subsequent studies have revealed that E2F exists in complexes with a variety of cellular proteins

including those encoded by the retinoblastoma tumor suppressor (RB) gene, p107, cyclin A, cyclin E and cdk2 (Schwarz et al., EMBO J. 12:1013-1020 (1993; Lees et al., Genes & Development 6: 1874-1885 (1992); Devoto et al., Cell 68: 167-176 (1992); and Pagano et al., Science 255:1146 (1992)).

It has been suggested that the E2F protein is a transcriptional regulator of genes containing E2F binding sites in their promotors, and that its interaction with the product of the RB gene affects its function by preventing E2F binding to the target sequence and/or altering E2F transcriptional activity by converting a positive into a negative signal (Hamel et al., Mol. Cell. Biol. 12: 3431-3438 (1992); Weintraub et al., Nature 358:259-261 (1992)). Sequences homologous to the E2F-binding site have been found upstream of cellular genes that encode proteins involved in the G_1-S transition and in DNA synthesis, including thymidine kinase, dehydrofolate reductase, DNA polymerase-α, cdc2, c-myc, N-myc, c-myb, B-myb and cyclin D1 (Nevins, Science 258:424-429 (1992); Lam and Watson, EMBO J. 12:2705-2713, 1993; Motokura and Arnold, Genes, Chromosome & Cancer 7:89-95, 1993). Although the importance of the E2F binding site for the regulation of the activity of these genes was demonstrated in several cases (Slansky et al., Mol. Cell. Biol. 13:1610-1618, (1993); Lam et al., EMBO J. 12:2705-2713 (1993), the functional and biochemical characteristics of the E2F protein were solely inferred based on its interaction with other cellular and viral factors rather than as a distinct protein.

Mammalian cDNA has been cloned encoding a protein with properties of authentic E2F, including sequence-specific DNA binding activity and binding to pRB (Shan et al., Mol. Cell. Biol. 12:5620-5631 (1992); Kaelin et

al., Cell 70:351-364 (1992); Helin et al., Cell 70: 337-350 (1992)).

The retinoblastoma tumor suppressor gene (RB) encompasses 180 kb of DNA mapping to chromosome 13q14. The RB gene encodes a 105-kDA nuclear phosphoprotein (pRB) which has been shown to play a key role in the regulation of cell division. The RB gene is ubiquitously expressed in vertebrates. In interphasic cells, pRB contributes to maintain the quiescent state of the cell by repressing transcription of genes required for the cell cycle through interaction with transcriptional factors such as E2F (Wagner and Green, Nature 353, 189-190, 1991; Nevins, Science 258, 424-429, 1992; Hiebert et al., Genes Develop. 6, 177-185, 1992). Loss of RB function is associated with the loss of cellular proliferative control. It has been further demonstrated that the loss of RB activity can induce cell transformation, as evidenced by the reversion of the transformed phenotype in pRB$^-$ cells after replacement of a functional pRB (Huang et al., Science 242, 1563-1565, 1988; Bookstein et al., Science 247, 712-715, 1990; Sumegi et al., Cell Growth Differ. 1, 247-250, 1990; Takahashi et al., Proc. Natl. Acad. Sci. USA 88, 5257-5261, 1991; Chen et al., Cell Growth Differ. 3, 119-125, 1992). The genetically reconstituted cells lost tumorigenicity when implanted into immunodeficient mice. Thus, RB is an authentic tumor suppressor.

Mutations of the RB gene which have lead to inactivation of its normal function have been found not only in 100% of retinoblastomas but also in other cancers, including small cell lung carcinoma (Harbour et al., Science 241, 353-357, 1988; Yokota et al., Oncogene 3, 471-475, 19889), osteosarcoma (Toguchida et al. Cancer Res. 48, 3939-3943, 1988), bladder carcinoma (Horowitz et al., Science 243, 937-940, 1989), prostate

carcinoma (Bookstein et al., Proc. Natl. Acad. Sci. USA 87, 7762-7766, 1990) and breast cancer (Lee et al., Science 241, 218-221, 1988).

E2F is a functional target for the action of the tumor suppressor protein pRB. The transcriptional activating capacity of E2F appears to be controlled by its interaction with RB, as well as a related protein termed p107 (Hiebert et al., Genes Dev. 6, 117, 1992; Nevins, Science 258, 424-429, 1992). RB can repress transcription of promotors that contain E2F-binding motifs (Hamel et al., Mol. Cell. Biol. 12, 3431-3438, 1992). It has been proposed that binding of RB to E2F inactivates E2F (Chellappan et al., Cell 65, 1053-1061, 1991). Alternatively, it has been suggested that the RB-E2F complex is an active complex that, when bound to the E2F site, inhibits the activity of other promotor elements and thus silences transcription (Weintraub et al., Nature 358, 259-261, 1992).

While it has been suggested that E2F is involved in the regulation of mammalian gene expression, the role of E2F in cell proliferation has not been established.

Summary of the Invention

The invention provides antisense oligonucleotides and pharmaceutical compositions thereof. A pharmaceutical composition comprises a pharmaceutically acceptable carrier and an antisense oligonucleotide specific for E2F-1 as hereinafter defined.

According to one embodiment, the oligonucleotide has a nucleotide sequence capable of forming a stable duplex with a portion of an mRNA transcript of the E2F-1 gene.

The oligonucleotide is generally at least an 8-mer oligonucleotide, that is, the oligonucleotide is an

oligomer containing at least 8 nucleotide residues, more preferably at least about 12 nucleotides. The preferred maximum size of the oligonucleotide is about 60 nucleotides, more preferably about 50 nucleotides. The oligomer is preferably an oligodeoxynucleotide. While oligonucleotides smaller than 12-mers may be utilized, they are statistically more likely to hybridize with non-targeted sequences, and for this reason may be less specific. In addition, a single mismatch may destabilize the hybrid. While oligonucleotides larger than 40-mers may be utilized, uptake may become more difficult without specialized vehicles or oligonucleotide carriers. Most preferably, the oligonucleotide is a 15- to 40-mer oligodeoxynucleotide, more advantageously an 18- to 30-mer.

While in principle oligonucleotides having a sequence complementary to any region of the E2F-1 mRNA find utility in the present invention, preferred are oligonucleotides capable of forming a stable duplex with a portion of the transcript lying within about 50 nucleotides (preferably within about 40 nucleotides) upstream (the 5' direction), or about 50 (preferably 40) nucleotides downstream (the 3' direction) from the translation initiation codon. Also preferred are oligonucleotides which are capable of forming a stable duplex with a portion of a E2F-1 mRNA transcript including the translation initiation codon.

The invention is also a method for inhibiting cell proliferation. The cells are contacted with an amount of E2F-1 antisense oligonucleotide effective for inhibiting proliferation. According to one preferred embodiment, the cells so treated comprise cancer cells. The invention thus provides a method of treating neoplastic disease in vivo or ex vivo comprising administering to an individual or cells harvested from

the individual an effective amount of E2F-1 antisense oligonucleotide. In particular, neoplastic diseases treatable include those diseases characterized by inactivation of the retinoblastoma tumor suppressor gene. Such diseases include, by way of example and not by way of limitation, glioblastoma, retinoblastoma, small cell lung cancer, osteosarcoma, bladder cancer, prostate cancer and breast cancer.

According to another embodiment, the invention is an artificially-constructed gene comprising a promotor segment and a segment containing E2F-1 DNA in inverted orientation such that transcription of the artificially-constructed gene produces RNA complementary to a portion of an mRNA transcript of the E2F-1 gene. The gene may be introduced into target cells to inhibit the proliferation of those cells. The artificially-constructed gene may be introduced into the target cells by, for example, transfection, transduction with a viral vector, or microinjection.

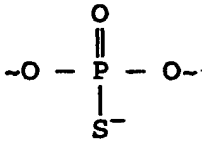
Definitions

An "antisense oligonucleotide specific for E2F-1" or "E2F-1 antisense oligonucleotide" is meant an oligonucleotide having a sequence (i) capable of forming a stable triplex with a portion of the E2F-1 gene, or (ii) capable of forming a stable duplex with a portion of an mRNA transcript of the E2F-1 gene.

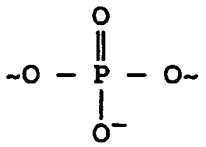
The term "oligonucleotide" as used herein includes linear oligomers of natural or modified monomers or linkages, including deoxyribonucleosides, ribonucleosides, α-anomeric forms thereof, polyamide nucleic acids, and the like, capable of specifically binding to a target polynucleotide by way of a regular pattern of monomer-to-monomer interactions, such as Watson-Crick type of base pairing, Hoogsteen or reverse Hoogsteen

types of base pairing, or the like. Usually, monomers are linked by phosphodiester bonds or analogs thereof to form oligonucleotides ranging in size from a few monomeric units, e.g., 3-4, to several hundreds of monomeric units. Analogs of phosphodiester linkages include: phosphorothioate, phosphorodithioate, phosphoroselenoate, phosphorodiselenoate, phosphoroanilothioate, phosphoranilidate, phosphoramidate, and the like, as more fully described below. As used herein, "nucleoside" includes the natural nucleosides, including 2'-deoxy and 2'-hydroxyl forms, e.g., as described in Kornberg and Baker, DNA Replication, 2nd Ed. (Freeman, San Francisco, 1992). "Analogs" in reference to nucleosides includes synthetic nucleosides having modified base moieties and/or modified sugar moieties, e.g., described generally by Scheit, Nucleotide Analogs (John Wiley, New York, 1980). Such analogs include synthetic nucleosides designed to enhance binding properties, e.g., duplex or triplex stability, specificity, or the like.

The term "phosphorothioate oligonucleotide" means an oligonucleotide wherein one or more of the internucleotide linkages is a phosphorothioate group,

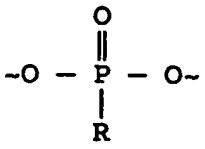


as opposed to the phosphodiester group



which is characteristic of unmodified oligonucleotides.

By "alkylphosphonate oligonucleoside" is meant an oligonucleotide wherein one or more of the internucleotide linkages is an alkylphosphonate group,



wherein R is an alkyl group, preferably methyl or ethyl.

"Stability" in reference to duplex or triplex formation roughly means how tightly an antisense oligonucleotide binds to its intended target sequence; more precisely, it means the free energy of formation of the duplex or triplex under physiological conditions. Melting temperature under a standard set of conditions, e.g., as described below, is a convenient measure of duplex and/or triplex stability. Preferably, antisense oligonucleotides of the invention are selected that have melting temperatures of at least 50°C under the standard conditions set forth below; thus, under physiological conditions and the preferred concentrations, duplex or triplex formation will be substantially favored over the state in which the antisense oligonucleotide and its target are dissociated. It is understood that a stable duplex or triplex may in some embodiments include mismatches between base pairs and/or among base triplets in the case of triplexes. Preferably, antisense oligonucleotides of the invention form perfectly matched duplexes and/or triplexes with their target polynucleotides.

The term "downstream" when used in reference to a direction along a nucleotide sequence means the 5′→3′ direction. Similarly, the term "upstream" means the 3′→5′ direction.

The term "E2F-1 mRNA transcript" means the presently known mRNA transcript of the E2F-1 gene and all variations thereof, or any further transcripts which may be elucidated.

By "retinoblastoma tumor suppressor gene inactivation" is meant the interruption of expression of functional protein corresponding to the nuclear phosphoprotein which is the product of the variously denominated "retinoblastoma gene", "retinoblastoma tumor suppressor gene", "retinoblastoma susceptibility gene" or "RB".

Description of the Figures

Fig. 1 is a determination, by S1 nuclease assay, of the levels of E2F-1 antisense transcripts and endogenous γ-actin mRNA after dexamethasone (DEX) treatment of E2F-1 antisense-transfected (TASA) and untransfected (T98G) human glioblastoma cells (lane 1, T98G cells; lane 2, T98G cells +DEX; lane 3, TASA cells; line 4, TASA cells -DEX).

Fig. 2 is a Western blot of the level of E2F-1 protein in T98G and TASA cells after serum stimulation and DEX induction. The cell lines were made quiescent and subsequently stimulated with 10% fetal calf serum (FCS) for 24 hours with and without DEX. Nuclear protein extracts (200 μg) were loaded onto a 10% acrylamide gel, and a Western blot was performed with an E2F-1-specific monoclonal antibody (lane 1, quiescent T98G cells; lane 2, quiescent TASA cells; lane 3, T98G cells+FCS; lane 4, T98G cells+FCS+DEX; lane 5, TASA cells+FCS; lane 6, TASA cells+FCS+DEX).

Fig. 3A is a cell cycle analysis of T98G cells and TASA cells after stimulation with serum and DEX. The percent of cells in the S phase as a function of time is given (-△-, T98G; -▲-, T98G+DEX; -□-, TASA; -■-, TASA+DEX).

Fig. 3B is similar to Fig. 3A, showing the percentage of T98G cells in the G_2 phase as a function of time (-▵-, T98G; -▲- T98G+DEX).

Fig. 3C is similar to Fig. 3B, showing the percentage of TASA cells in the G_2 phase as a function of time (-□-, TASA; -■- TASA+DEX).

Fig. 4 is a band-shift analysis of double-stranded oligonucleotides representative of various promotors and the recombinant glutathione S-transferase (GST)-E2F recombinant protein (lanes 1 and 2, E2 promotor; lines 3 and 4, DNA polymerase-α promotor; lanes 5 and 6, cdc2 promotor; lines 7 and 8, cyclin D1 promotor; lines 9 and 10, c-myb promotor). Odd and even numbers in Fig. 4 correspond to +GST and +E2F, respectively.

Fig. 5A represents a transient transfection assay of human T98G glioblastoma cells cotransfected with CMV E2F-1 expression vector and various constructs comprising different human promotors linked to the chloramphenicol acetyltransferase (CAT) gene as a reporter gene. Transactivation of the different constructs by E2F-1 is indicated with respect to basal CAT activity after 48 hours.

Fig. 5B schematically represents the promotor constructs used in the Fig. 5A study (shaded boxes: CAT gene; open boxes: nucleotide positions of different E2F sites).

Fig. 6A represents the results of a transient transfection assay of human T98G glioblastoma cells transfected with the PstI-NcoI mutant c-myb promotor fragment P1mut-CAT (Mutant) or P1-CAT wild type c-myb promotor (Wild Type), with (+E2F) or without (control) cotransfection of CMV-E2F-1. The P1mut-CAT fragment bears a mutation at the E2F binding site. Transactivation is indicated with respect to CAT activity.

Fig. 6B records the ^{14}C radioactivity of the isolated spots shown in Fig. 6A, as measured in a scintillation counter. In repeated experiments, the activity of the mutant promotor upon transactivation by E2F-1 was approximately 30% that of the wild type promotor.

Detailed Description of the Invention

The cDNA nucleotide sequence of the E2F-1 gene and predicted 476 amino acid polypeptide are provided by Shan et al., Mol. Cell. Biol. 12, 5620-5631 (1992), Kaelin et al., Cell 70, 351-364 (1992), and Helin et al., Cell 70, 337-350 (1992), the entire disclosures of which are incorporated herein by reference. The translation initiation codon ATG is preceded by a 5'-untranslated region. The termination codon TGA is followed by a 3'-untranslated region spanning about 985 nucleotides, including a polyadenylation sequence at the 3' end.

It has now been demonstrated that the E2F-1 protein plays a role in the control of cell proliferation. In particular, it appears that E2F-1 plays a major role in the control of cell cycle progression via transcriptional regulation of proliferation-associated genes. Direct evidence is provided herein that E2F-1 is, at least partially, required in the S phase of the cell cycle in human cells. The induction of antisense E2F-1 results in a marked delay in the completion of DNA synthesis and completion of the cell cycle S phase. Furthermore, it is shown herein that recombinant E2F-1 protein binds to and transactivates human promotors, including the DNA polymerase-α, c-myb and cyclin D1 promotors. The importance of these genes in the control of cellular proliferation has been extensively demonstrated (Campbell, Annu. Rev. Biochem. 55: 733-

771, 1986; Gewirtz and Calabretta, Science 242:1303-1306, 1988; Sala and Calabretta, Proc. Natl. Acad. Sci. USA 89: 10415-10419, 1992; Baldin et al., Genes & Development 7:812-821, 1993; Quelle et al., Genes and Development 7: 1559-1571, 1993; and Ewen et al. Cell 73;487-497, 1993). The results described herein suggest that E2F-1 acts as a master regulator of a series of genes involved in the G_1-S transition of the cell cycle and DNA synthesis.

Antisense oligonucleotides specific for E2F-1 are therefor useful for inhibiting undesirable cell proliferation, such as the proliferation of cancer cells. In particular, E2F-1 antisense oligonucleotides are believed useful in the treatment of disorders characterized by the loss of functional RB. The loss of RB suppression of E2F transcriptional activating capacity, as occurs in retinoblastoma and other cancers characterized by RB-inactivating mutations, may be compensated through down-regulation of E2F expression by antisense inhibition. The loss of RB activity is associated with all retinoblastomas, and some occurrences of small cell lung cancer, glioblastoma, osteosarcoma, bladder cancer, prostate cancer and breast cancer. The E2F-1 antisense oligonucleotides of the invention are thus believed to be particularly useful in the treatment of the disorders.

Whether or not a particular cancer involves an RB mutation and loss of function pRB may be ascertained by conventional molecular biological techniques, such as by sequencing of the patient's RB gene to determine whether an inactivating mutation is present. Alternatively, loss of RB function may be identified by a loss of the pRB protein's ability to bind E2F-1, as may be indicated by an E2F-1 binding assay. Such assays are described by Shan et al., Mol. Cell. Biol. 12:5620-5631

(1992), Kaelin et al., Cell 70:351-364 and Helin et al., Cell 70:337-350 (1992).

In the practice of the present invention, target E2F-1 polynucleotides may be single-stranded or double-stranded DNA or RNA; however, single-stranded DNA or RNA targets are preferred. It is understood that the target to which the E2F-1 antisense oligonucleotides of the invention are directed include allelic forms of the E2F-1 gene and mRNA. There is substantial guidance in the literature for selecting particular sequences for antisense oligonucleotides given a knowledge of the sequence of the target polynucleotide, e.g., Peyman and Ulmann, Chemical Reviews, 90:543-584, 1990; Crooke, Ann. Rev. Pharmacal. Toxicol., 32:329-376 (1992); and Zamecnik and Stephenson, Proc. Natl. Acad. Sci., 75:280-284 (1974). Preferably, the sequences of E2F-1 antisense compounds are selected such that the G-C content is at least 60%. Preferred mRNA targets include the 5' cap site, tRNA primer binding site, the initiation codon site, the mRNA donor splice site, and the mRNA acceptor splice site, e.g., Goodchild et al., U.S. patent 4,806,463.

Where the target polynucleotide comprises the E2F-1 mRNA transcript, oligonucleotides complementary to and hybridizable with any portion of the transcript are, in principle, effective for inhibiting translation, and capable of inducing the effects herein described. It is believed that translation is most effectively inhibited by blocking the mRNA at a site at or near the initiation codon. Thus, oligonucleotides complementary to the 5'-region of the E2F-1 mRNA transcript are preferred. Oligonucleotides complementary to the E2F-1 mRNA, including the initiation codon (the first codon at the 5' end of the translated

portion of the E2F-1 transcript), or codons adjacent the initiation codon, are preferred.

While antisense oligomers complementary to the 5'-region of the E2F-1 transcript are preferred, particularly the region including the initiation codon, it should be appreciated that useful antisense oligomers are not limited to those oligomers complementary to the sequences found in the translated portion of the mRNA transcript, but also includes oligomers complementary to nucleotide sequences contained in, or extending into, the 5'- and 3'-untranslated regions.

According to a preferred embodiment of the invention, the antisense oligonucleotide is complementary to the mRNA transcript beginning with the initiation codon of the transcript and extending downstream thereof for a distance of 50 nucleotides.

Smaller oligomers based upon the 50-mer sequence, in particular, oligomers hybridizable to segments of the E2F-1 message containing the initiation codon, may be utilized. Particularly preferred are oligomers containing at least 12 nucleotides.

Antisense oligonucleotides of the invention may comprise any polymeric compound capable of specifically binding to a target polynucleotide by way of a regular pattern of monomer-to-nucleoside interactions, such as Watson-Crick type of base pairing, Hoogsteen or reverse Hoogsteen types of base pairing, or the like.

Antisense compounds of the invention may also contain pendent groups or moieties, either as part of or separate from the basic repeat unit of the polymer, to enhance specificity, nuclease resistance, delivery, or other property related to efficacy, e.g., cholesterol moieties, duplex intercalators such as acridine, poly-L-lysine, "end-capping" with one or more nuclease-

resistant linkage groups such as phosphorothioate, and the like.

For example, it is known that enhanced lipid solubility and/or resistance to nuclease digestion results by substituting an alkyl group or alkoxy group for a phosphate oxygen in the internucleotide phosphodiester linkage to form an alkylphosphonate oligonucleoside or alkylphosphotriester oligonucleotide. Non-ionic oligonucleotides such as these are characterized by increased resistance to nuclease hydrolysis and/or increased cellular uptake, while retaining the ability to form stable complexes with complementary nucleic acid sequences. The alkylphosphonates, in particular, are stable to nuclease cleavage and soluble in lipid. The preparation of alkylphosphonate oligonucleosides is disclosed in Tso et al., U.S. patent 4,469,863.

Preferably, nuclease resistance is conferred on the antisense compounds of the invention by providing nuclease-resistant internucleosidic linkages. Many such linkages are known in the art, e.g., phosphorothioate: Zon and Geiser, Anti-Cancer Drug Design, 6:539-568 (1991); Stec et al., U.S. patent 5,151,510; Hirschbein, U.S. patent 5,166,387; Bergot, U.S. patent 5,183,885; phosphorodithioates: Marshall et al., Science, 259:1564-1570 (1993); Caruthers and Nielsen, International application PCT/US89/02293; phosphoramidates, e.g., $-OP(=O)(NR^1R^2)-O-$ with R^1 and R^2 hydrogen or C_1-C_3 alkyl; Jager et al., Biochemistry, 27:7237-7246 (1988); Froehler et al., International application PCT/US90/03138; peptide nucleic acids: Nielsen et al., Anti-Cancer Drug Design, 8: 53-63 (1993), International application PCT/EP92/01220; methylphosphonates: Miller et al., U.S. patent 4,507,433, Ts'o et al., U.S. patent 4,469,863; Miller et al., U.S. patent 4,757,055; and P-

chiral linkages of various types, especially phosphorothioates, Stec et al., European patent application 506,242 (1992) and Lesnikowski, Bioorganic Chemistry, 21:127-155 (1993). Additional nuclease linkages include phosphoroselenoate, phosphorodiselenoate, phosphoroanilothioate, phosphoranilidate, alkylphosphotriester such as methyl- and ethylphosphotriester, carbonate such as carboxymethyl ester, carbamate, morpholino carbamate, 3'-thioformacetal, silyl such as dialkyl(C_1-C_6)- or diphenylsilyl, sulfamate ester, and the like. Such linkages and methods for introducing them into oligonucleotides are described in many references, e.g., reviewed generally by Peyman and Ulmann, Chemical Reviews 90:543-584 (1990); Milligan et al., J. Med. Chem., 36:1923-1937 (1993); Matteucci et al., International application PCT/US91/06855.

Resistance to nuclease digestion may also be achieved by modifying the internucleotide linkage at both the 5' and 3' termini with phosphoroamidites according to the procedure of Dagle et al., Nucl. Acids Res. 18, 4751-4757 (1990).

Preferably, phosphorus analogs of the phosphodiester linkage are employed in the compounds of the invention, such as phosphorothioate, phosphorodithioate, phosphoramidate, or methylphosphonate. More preferably, phosphorothioate is employed as the nuclease resistant linkage.

Phosphorothioate oligonucleotides contain a sulfur-for-oxygen substitution in the internucleotide phosphodiester bond. Phosphorothioate oligonucleotides combine the properties of effective hybridization for duplex formation with substantial nuclease resistance, while retaining the water solubility of a charged phosphate analogue. The charge is believed to confer the

property of cellular uptake via a receptor (Loke et al., Proc. Natl. Acad. Sci., 86, 3474-3478 (1989)).

It is understood that in addition to the preferred linkage groups, compounds of the invention may comprise additional modifications, e.g., boronated bases, Spielvogel et al., 5,130,302; cholesterol moieties, Shea et al., Nucleic Acids Research, 18:3777-3783 (1990) or Letsinger et al., Proc. Natl. Acad. Sci., 86:6553-6556 (1989); and 5-propynyl modification of pyrimidines, Froehler et al., Tetrahedron Lett., 33:5307-5310 (1992).

Preferably, antisense compounds of the invention are synthesized by conventional means on commercially available automated DNA synthesizers, e.g., an Applied Biosystems (Foster City, CA) model 380B, 392 or 394 DNA/RNA synthesizer. Preferably, phosphoramidite chemistry is employed, e.g., as disclosed in the following references: Beaucage and Iyer, Tetrahedron, 48:2223-2311 (1992); Molko et al., U.S. patent 4,980,460; Koster et al., U.S. patent 4,725,677; Caruthers et al., U.S. patents 4,415,732; 4,458,066; and 4,973,679.

In embodiments where triplex formation is desired, there are constraints on the selection of target sequences. Generally, third strand association via Hoogsteen type of binding is most stable along homo-pyrimidine-homopurine tracks in a double stranded target. Usually, base triplets form in T-A*T or C-G*C motifs (where "-" indicates Watson-Crick pairing and "*" indicates Hoogsteen type of binding); however, other motifs are also possible. For example, Hoogsteen base pairing permits parallel and antiparallel orientations between the third strand (the Hoogsteen strand) and the purine-rich strand of the duplex to which the third strand binds, depending on conditions and the

composition of the strands. There is extensive guidance in the literature for selecting appropriate sequences, orientation, conditions, nucleoside type (e.g., whether ribose or deoxyribose nucleosides are employed), base modifications (e.g., methylated cytosine, and the like) in order to maximize, or otherwise regulate, triplex stability as desired in particular embodiments, e.g., Roberts et al., Proc. Natl. Acad. Sci., 88:9397-9401 (1991); Roberts et al., Science, 258:1463-1466 (1992); Distefano et al., Proc. Natl. Acad. Sci., 90:1179-1183 (1993); Mergny et al., Biochemistry, 30:9791-9798 (1992); Cheng et al., J. Am. Chem. Soc., 114:4465-4474 (1992); Beal and Dervan, Nucleic Acids Research, 20:2773-2776 (1992); Beal and Dervan, J. Am. Chem. Soc., 114:4976-4982; Giovannangeli et al., Proc. Natl. Acad. Sci., 89:8631-8635 (1992); Moser and Dervan, Science, 238:645-650 (1987); McShan et al., J. Biol. Chem., 267: 5712-5721 (1992); Yoon et al., Proc. Natl. Acad. Sci., 89:3840-3844 (1992); and Blume et al., Nucleic Acids Research, 20:1777-1784 (1992).

The length of the oligonucleotide moieties is sufficiently large to ensure that specific binding will take place only at the desired target polynucleotide and not at other fortuitous sites, as explained in many references, e.g., Rosenberg et al., International application PCT/US92/05305; or Szostak et al., Meth. Enzymol, 68:419-429 (1979). The upper range of the length is determined by several factors, including the inconvenience and expense of synthesizing and purifying oligomers greater than about 30-40 nucleotides in length, the greater tolerance of longer oligonucleotides for mismatches than shorter oligonucleotides, whether modifications to enhance binding or specificity are present, whether duplex or

triplex binding is desired, and the like. Usually, antisense compounds of the invention have lengths in the range of about 12 to 60 nucleotides. More preferably, antisense compounds of the invention have lengths in the range of about 15 to 40 nucleotides; and most preferably, they have lengths in the range of about 18 to 30 nucleotides.

In general, the antisense oligonucleotides used in the practice of the present invention will have a sequence which is completely complementary to a selected portion of the target polynucleotide. Absolute complementarity is not however required, particularly in larger oligomers. Thus, reference herein to a "nucleotide sequence complementary to" a target polynucleotide does not necessarily mean a sequence having 100% complementarity with the target segment. In general, any oligonucleotide having sufficient complementarity to form a stable duplex with the target (e.g. the E2F-1 mRNA) that is, an oligonucleotide which is "hybridizable", is suitable. Stable duplex formation depends on the sequence and length of the hybridizing oligonucleotide and the degree of complementarity with the target polynucleotide. Generally, the larger the hybridizing oligomer, the more mismatches may be tolerated. More than one mismatch probably will not be tolerated for antisense oligomers of less than about 21 nucleotides. One skilled in the art may readily determine the degree of mismatching which may be tolerated between any given antisense oligomer and the target sequence, based upon the melting point, and therefore the thermal stability, of the resulting duplex.

Preferably, the thermal stability of hybrids formed by the antisense oligonucleotides of the invention are determined by way of melting, or strand

dissociation, curves. The temperature of fifty percent strand dissociation is taken as the melting temperature, T_m, which, in turn, provides a convenient measure of stability. T_m measurements are typically carried out in a saline solution at neutral pH with target and antisense oligonucleotide concentrations at between about 1.0-2.0 μM. Typical conditions are as follows: 150 mM NaCl and 10mM $MgCl_2$ in a 10 mM sodium phosphate buffer (pH 7.0) or in a 10mM Tris-HCl buffer (pH 7.0). Data for melting curves are accumulated by heating a sample of the antisense oligonucleotide/target polynucleotide complex from room temperature to about 85-90°C. As the temperature of the sample increases, absorbance of 260 nm light is monitored at 1°C intervals, e.g., using a Cary (Australia) model 1E or a Hewlett-Packard (Palo Alto, CA) model HP 8459 UV/VIS spectrophotometer and model HP 89100A temperature controller, or like instruments. Such techniques provide a convenient means for measuring and comparing the binding strengths of antisense oligonucleotides of different lengths and compositions.

Pharmaceutical compositions of the invention include a pharmaceutical carrier that may contain a variety of components that provide a variety of functions, including regulation of drug concentration, regulation of solubility, chemical stabilization, regulation of viscosity, absorption enhancement, regulation of pH, and the like. The pharmaceutical carrier may comprise a suitable liquid vehicle or excipient and an optional auxiliary additive or additives. The liquid vehicles and excipients are conventional and commercially available. Illustrative thereof are distilled water, physiological saline, aqueous solutions of dextrose, and the like. For water soluble formulations, the pharmaceutical composition

preferably includes a buffer such as a phosphate buffer, or other organic acid salt, preferably at a pH of between about 7 and 8. For formulations containing weakly soluble antisense compounds, micro-emulsions may be employed, for example by using a nonionic surfactant such as polysorbate 80 in an amount of 0.04-0.05% (w/v), to increase solubility. Other components may include antioxidants, such as ascorbic acid, hydrophilic polymers, such as, monosaccharides, disaccharides, and other carbohydrates including cellulose or its derivatives, dextrins, chelating agents, such as EDTA, and like components well known to those in the pharmaceutical sciences, e.g., Remington's Pharmaceutical Science, latest edition (Mack Publishing Company, Easton, PA).

Antisense compounds of the invention include the pharmaceutically acceptable salts thereof, including those of alkaline earths, e.g., sodium or magnesium, ammonium or NX_4^+, wherein X is C_1-C_4 alkyl. Other pharmaceutically acceptable salts include organic carboxylic acids such as acetic, lactic, tartaric, malic, isethionic, lactobionic, and succinic acids; organic sulfonic acids such as methanesulfonic, ethanesulfonic, and benzenesulfonic; and inorganic acids such as hydrochloric, sulfuric, phosphoric, and sulfamic acids. Pharmaceutically acceptable salts of a compound having a hydroxyl group include the anion of such compound in combination with a suitable cation such as Na^+, NH_4^+, or the like.

The E2F-1 antisense oligonucleotides are preferably administered parenterally, most preferably intravenously. The vehicle is designed accordingly. Alternatively, oligonucleotide may be administered subcutaneously via controlled release dosage forms.

In addition to administration with conventional carriers, the antisense oligonucleotides may be administered by a variety of specialized oligonucleotide delivery techniques. Sustained release systems suitable for use with the pharmaceutical compositions of the invention include semi-permeable polymer matrices in the form of films, microcapsules, or the like, comprising polylactides, copolymers of L-glutamic acid and gamma-ethyl-L-glutamate, poly(2-hydroxyethyl methacrylate), and like materials, e.g., Rosenberg et al., International application PCT/US92/05305.

The oligonucleotides may be encapsulated in liposomes for therapeutic delivery, as described for example in Liposome Technology, Vol. II, Incorporation of Drugs, Proteins, and Genetic Material, CRC Press. The oligonucleotide, depending upon its solubility, may be present both in the aqueous layer and in the lipidic layer, or in what is generally termed a liposomic suspension. The hydrophobic layer, generally but not exclusively, comprises phospholipids such as lecithin and sphingomyelin, steroids such as cholesterol, ionic surfactants such as diacetylphosphate, stearylamine, or phosphatidic acid, and/or other materials of a hydrophobic nature. The use of liposomes to introduce nucleotides into cells is described in U.S. Pat. Nos. 4,897,355 and 4,394,448, for example. For general methods of preparing liposomes comprising biological materials see, for example, U.S. Pat. Nos. 4,235,871, 4,231,877, 4,224,179, 4,753,788, 4,673,657, 4,247,411 and 4,814,270.

The oligonucleotides may be conjugated to poly(L-lysine) to increase cell penetration. Such conjugates are described by Lemaitre et al., Proc. Natl. Acad. Sci. USA, 84, 648-652 (1987). The procedure requires that the 3'-terminal nucleotide be a ribonucleotide.

The resulting aldehyde groups are then randomly coupled to the epsilon-amino groups of lysine residues of poly(L-lysine) by Schiff base formation, and then reduced with sodium cyanoborohydride. This procedure converts the 3'-terminal ribose ring into a morpholine structure antisense oligomers.

Antisense compounds of the invention also include conjugates of such oligonucleotides with appropriate ligand-binding molecules. The oligonucleotides may be conjugated for therapeutic administration to ligand-binding molecules which recognize cell-surface molecules, such as according to International Patent Application WO 91/04753. The ligand-binding molecule may comprise, for example, an antibody against a cell surface antigen, an antibody against a cell surface receptor, a growth factor having a corresponding cell surface receptor, an antibody to such a growth factor, or an antibody which recognizes a complex of a growth factor and its receptor. Methods for conjugating ligand-binding molecules to oligonucleotides are detailed in WO 91/04753.

In particular, the growth factor to which the antisense oligonucleotide may be conjugated, may comprise transferrin or folate. Transferrin-polylysine-oligonucleotide complexes or folate-polylysine-oligonucleotide complexes may be prepared for uptake by cells expressing high levels of transferrin or folate receptor. The preparation of transferrin complexes as carriers of oligonucleotide uptake into cells is described by Wagner et al., Proc. Natl. Acad. Sci. USA 87, 3410-3414 (1990). Inhibition of leukemia cell proliferation by transferrin receptor-mediated uptake of c-myb antisense oligonucleotides conjugated to transferrin has been demonstrated by Citro et al., Proc. Natl. Acad. Sci. USA., 89, 7031-7035 (1992).

Cellular delivery of folate-macromolecule conjugates via folate receptor endocytosis, including delivery of an antisense oligonucleotide, is described by Low et al., U.S. Patent 5,108,921. Also see, Leamon et al., Proc. Natl. Acad. Sci. 88, 5572 (1991).

A preferred method of administration of oligonucleotide comprises either systemic or regional perfusion, as is appropriate. According to a method of regional perfusion, the afferent and efferent vessels supplying the extremity containing the lesion are isolated and connected to a low-flow perfusion pump in continuity with an oxygenator and a heat exchanger. The iliac vessels may be used for perfusion of the lower extremity. The axillary vessels are cannulated high in the axilla for upper extremity lesions. Oligonucleotide is added to the perfusion circuit, and the perfusion is continued for an appropriate time period, e.g., one hour. Perfusion rates of from 100 to 150 ml/minute may be employed for lower extremity lesions, while half that rate should be employed for upper extremity lesions. Systemic heparinization may be used throughout the perfusion, and reversed after the perfusion is complete. This isolation perfusion technique permits administration of higher doses of chemotherapeutic agent than would otherwise be tolerated upon infusion into the arterial or venous systemic circulation.

For systemic infusion, the oligonucleotides are preferably delivered via a central venous catheter, which is connected to an appropriate continuous infusion device. Indwelling catheters provide long term access to the intravenous circulation for frequent administration of drugs over extended time periods. They are generally surgically inserted into the external cephalic or internal jugular vein under general or

local anesthesia. The subclavian vein is another common site of catheterization. The infuser pump may be external, or may form part of an entirely implantable central venous system such as the INFUSAPORT system available from Infusaid Corp., Norwood, MA and the PORT-A-CATH system available from Pharmacia Laboratories, Piscataway, NJ. These devices are implanted into a subcutaneous pocket under local anesthesia. A catheter, connected to the pump injection port, is threaded through the subclavian vein to the superior vena cava. The implant contains a supply of oligonucleotide in a reservoir which may be replenished as needed by injection of additional drug from a hypodermic needle through a self-sealing diaphragm in the reservoir. Completely implantable infusers are preferred, as they are generally well accepted by patients because of the convenience, ease of maintenance and cosmetic advantage of such devices.

As an alternative to treatment with exogenous oligonucleotide, antisense oligonucleotide synthesis may be induced in situ by local treatment of the targeted cells with a vector containing an artificially-constructed gene comprising a transcriptional promotor and E2F-1 DNA in inverted orientation. The E2F-1 DNA for insertion into the artificial gene in inverted orientation comprises cDNA which may be prepared, for example, by reverse transcriptase polymerase chain reaction from RNA using primers derived from the published E2F-1 cDNA sequence. Upon transcription, the inverted E2F-1 gene segment, which is complementary to the E2F-1 mRNA, is produced in situ in the targeted cell. The endogenously produced RNA hybridizes to E2F-1 mRNA, resulting in interference with E2F-1 function and inhibition of the proliferation of the targeted cell.

The promotor segment of the artificially-constructed gene serves as a signal conferring expression of the inverted E2F-1 sequence which lies downstream thereof. It will include all of the signals necessary for initiating transcription of the sequence. The promotor may be of any origin as long as it specifies a rate of transcription which will produce sufficient antisense mRNA to inhibit the expression of the E2F-1 gene, and therefore the proliferation of the targeted cells. Preferably, a highly efficient promotor such as a viral promotor is employed. Other sources of potent promotors include cellular genes that are expressed at high levels. The promotor segment may comprise a constitutive or a regulatable promotor.

The artificial gene may be introduced by any of the methods described in U.S. Patent 4,740,463, incorporated herein by reference. One technique is transfection, which can be done by several different methods. One method of transfection involves the addition of DEAE-dextran to increase the uptake of the naked DNA molecules by a recipient cell. See McCutchin, J.H. and Pagano, J.S., J. Natl. Cancer Inst. 41, 351-7 (1968). Another method of transfection is the calcium phosphate precipitation technique which depends upon the addition of Ca^{++} to a phosphate-containing DNA solution. The resulting precipitate apparently includes DNA in association with calcium phosphate crystals. These crystals settle onto a cell monolayer; the resulting apposition of crystals and cell surface appears to lead to uptake of the DNA. A small proportion of the DNA taken up becomes expressed in a transfectant, as well as in its clonal descendants. See Graham, F.L. and van der Eb, A.J., Virology 52, 456-467 (1973) and Virology 54, 536-539 (1973).

Transfection may also be carried out by cationic phospholipid-mediated delivery. In particular, polycationic liposomes can be formed from N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA). See Felgner et al., Proc. Natl. Acad. Sci., 84, 7413-7417 (1987) (DNA-transfection); Malone et al., Proc. Natl. Acad. Sci., 86, 6077-6081 (1989) (RNA-transfection).

Alternatively, the artificially-constructed gene can be introduced in to cells, in vitro or in vivo, via a transducing viral vector. See Tabin et al., Mol. Cel. Biol. 2, 426-436 (1982). Use of a retrovirus, for example, will infect a variety of cells and cause the artificial gene to be inserted into the genome of infected cells. Such infection could either be accomplished with the aid of a helper retrovirus, which would allow the virus to spread through the organism, or the antisense retrovirus could be produced in a helper-free system, such as ψ2-like cells (See Mann et al., Cell 33, 153-160, 1983) that package amphotropic viruses. A helper-free virus might be employed to minimize spread throughout the organism. Viral vectors in addition to retroviruses can also be employed, such as papovaviruses, SV40-like viruses, or papilloma viruses. The use of retroviruses for gene transfer has been reviewed by Eglitis and Anderson, BioTechniques 6, 608-614 (1988).

Vesicle fusion could also be employed to deliver the artificial gene. Vesicle fusion may be physically targeted to the malignant cells if the vesicle were approximately designed to be taken up by those cells. Such a delivery system would be expected to have a lower efficiency of integration and expression of the artificial gene delivered, but would have a higher specificity than a retroviral vector. A combination

strategy of targeted vesicles containing papilloma virus or retrovirus DNA molecules might provide a method for increasing the efficiency of expression of targeted molecules.

Particulate systems and polymers for in vitro and in vivo delivery of polynucleotides were extensively reviewed by Felgner in Advanced Drug Delivery Reviews 5, 163-187 (1990). Techniques for direct delivery of purified genes in vivo, without the use of retroviruses, has been reviewed by Felgner in Nature 349, 351-352 (1991). Such methods of direct delivery of polynucleotides may be utilized for local delivery of either exogenous E2F-1 antisense oligonucleotide or artificially-constructed genes producing E2F-1 antisense oligonucleotide in situ.

Recently, Wolf et al. demonstrated that direct injection of non-replicating gene sequences in a non-viral vehicle is possible. See Science, 247, 1465-1468 (1990). DNA injected directly into mouse muscle did not integrate into the host genome, and plasmid essentially identical to the starting material was recovered from the muscle months after injection. Interestingly, no special delivery system is required. Simple saline or sucrose solutions are sufficient to delivery DNA and RNA.

The E2F-1 antisense oligonucleotides may be used as the primary therapeutic for the treatment of the disease state, or may be used in combination with non-oligonucleotide agents.

For systemic or regional in vivo administration, the amount of antisense oligonucleotide may vary depending on the nature and extent of the neoplasm, the particular oligonucleotide utilized, and other factors. The actual dosage administered may take into account the size and weight of the patient, whether the nature

of the treatment is prophylactic or therapeutic in nature, the age, health and sex of the patient, the route of administration, whether the treatment is regional or systemic, and other factors. Intercellular concentrations of from about 1 to about 200 μg/ml at the target polynucleotide may be employed, preferably from about 10 μg/ml to about 100 μg/ml. The patient should receive a sufficient daily dosage of antisense oligonucleotide to achieve these intercellular concentrations of drug. The daily dosage may range from about 0.2 mg, more preferably from about 25 mg, to about 2 grams per day, with at least about 250 mg per day being preferred. An effective human continuous intravenous infusion dosage, based upon animal studies employing antisense oligonucleotides targeting other genes in antileukemic therapy, is about 0.4 mg/kg/day. Greater or lesser amounts of oligonucleotide may be administered, as required. Those skilled in the art should be readily able to derive appropriate dosages and schedules of administration to suit the specific circumstance and needs of the patient. It is believed that a course of treatment may advantageously comprise infusion of the recommended daily dose as a continuous intravenous infusion over 7 days. The oligonucleotide may be given for a period of from about 3 to about 28 days, more preferably from about 7 to about 10 days. Those skilled in the art should readily be able to determine the optimal dosage in each case. For modified oligonucleotides, such as phosphorothioate oligonucleotides, which have a half life of from 24 to 48 hours, the treatment regimen may comprise dosing on alternate days.

The antisense oligonucleotides may be used ex vivo, to purge neoplastic cells harvested from the patient to be later returned to the patient. For such

ex vivo antineoplastic application, the E2F-1 antisense oligonucleotides may be administered in amounts effective to kill neoplastic cells. Such amounts may vary depending on the particular oligonucleotide utilized, the relative sensitivity of the neoplastic cells to the oligonucleotide, and other factors. Concentrations from about 10 to 200 μg/ml per 10^5 cells may be employed, preferably from about 40 to 150 μg/ml per 10^5 cells. Supplemental dosing of the same or lesser amounts of oligonucleotide are advantageous to optimize the treatment.

For anticancer therapy, the effectiveness of the treatment may be assessed by routine methods which are used for determining whether or not remission has occurred. Such methods generally depend upon some combination of morphological, cytochemical, cytogenetic, immunologic and molecular analyses. In addition, remission can be assessed genetically by probing the level of expression of one or more relevant oncogenes. The reverse transcriptase polymerase chain reaction methodology can be used to detect even very low numbers of mRNA transcript. For example, RT-PCR has been used to detect and genotype the three known bcr-abl fusion sequences in Ph^1 leukemias. See PCT/US92/05035 and Kawasaki et al., Proc. Natl. Acad. Sci. USA 85, 5698-5702 (1988).

Typically, therapeutic success is assessed by the decrease and the extent of the primary and any metastatic diseases lesions. For solid tumors, decreasing tumor size is the primary indicia of successful treatment. Neighboring tissues should be biopsied to determine the extent to which metastasis has occurred. Tissue biopsy methods are known to those skilled in the art. More recent methods for detecting cancer cells have focused on detecting the presence of the gene for

the relevant oncogene, or its corresponding mRNA. See for example the following U.S. Patents: 4,681,840, 4,857,466 and 4,874,853. The presence of even a few copies of the target oncogene can be effectively detected by amplification using reverse transcriptase polymerase chain reaction technology. For a detailed discussion of such methods, see for example, Cancer: Principles & Practice of Oncology, edited by V. T. DeVita, S. Hellman and S.A. Rosenberg, J.B. Lippincott Company, Philadelphia, PA (3rd ed., 1989). Methods for diagnosing and monitoring the progress of neoplastic disorders vary depending upon the nature of the particular disease.

The practice of the invention is illustrated by the following non-limiting examples.

Example 1

Transfection of Human Glioblastoma Cell To Express E2F Antisense Transcript.

A. Preparation of E2F-1 Antisense Construct.

A vector pMAME2FAS1 expressing an antisense E2F-1 transcript, was obtained as follows. The human E2F-1 corresponding to the region from +581 to +1460 was amplified by reverse transcription-polymerase chain reaction (RT-PCR) using the 5' primer GTGTCGTCGA CCTGAACTG (SEQ ID NO:1) and the 3' primer TCCAAGCCCT GTCAGAAATC (SEQ ID NO:2) from the human glioblastoma T98G cell line using standard procedures. The 880-base pair fragment was cloned into the PCR II cloning vector (InVitrogen) and recombinant clones were sequenced. Clone p2E2F2 was found 100% identical to the published E2F-1 sequence in the same orientation with respect to the T7 promotor. An XhoI fragment was then cloned into

the pMAM-NEO vector (Clontech Laboratories, Inc., Pao Alto, CA). Sequence analysis of clone pMAME2FAS1 showed that it was in the antisense orientation with respect to the mouse mammary tumor virus long-terminal repeat (MMTV LTR).

B. Transfection of Glioblastoma Cells with E2F-1 Antisense Construct.

T98G human glioblastoma cells were cultured in Eagle's minimal essential medium containing 10% fetal calf serum (FCS) (GIBCO) as described by Neareer, Proc. Intl. Sci. Acad. USA 87, 6166-6170 (1990). Cells were transfected with plasmid pMAME2FAS1 using the calcium-phosphate precipitation method as described by Nicolaides et al., J. Biol. Chem. 267, 19665-19675 (1992). After twelve days of selection in culture medium containing 0.5 mg/ml of G418 (GIBCO), colonies were pooled and maintained in the presence of 0.2 mg/ml of G418. After twelve days of selection in the G418-containing medium, a cell line (TASA) was established from the pool of colonies. The TASA conditionally express the E2F-1 antisense transcript.

C. Measurement of Steady State E2F-1 Antisense Transcript Level by S1 Nuclease Assay.

S1 nuclease analysis was employed to measure steady-state levels of antisense E2F-1 expression in the TASA cell line before and after dexamethasone (DEX) treatment. Exponentially growing parental (T98G) and antisense E2F1-transfected (TASA) cells were stimulated with 1 μM DEX. Total cellular RNA was extracted after 12 hours as described by Chomczynski and Sacchi, Anal. Biochem. 162, 156-159 (1987). An S1 protection assay was performed as follows. A 973 base pair E2F-1 probe

was generated by digesting the p2E2F2 plasmid with NotI and HindIII. A γ-actin probe was generated by digesting the pActin plasmid with HindIII to produce a 1.3 kilobase fragment. Both fragments were dephosphorylated with calf intestinal phosphatase and end-labeled using [γ^{32}P]ATP and T4 polynucleotide kinase. Total cellular RNA (40 μg) was incubated with 10^4 cpm of probe for 5 min. at 90°C and hybridized for 16 hours in formamide buffer. S1 digestion was carried out for one hour at room temperature. Samples were electrophoresed on an 8% sequencing gel. The results are shown in Fig. 1 (lane 1, T98G cells; lane 2, T98G cells +DEX; lane 3, TASA cells; lane 4, TASA cells -DEX). The results indicate strong induction of the E2F-1 antisense transcripts upon DEX induction of the E2F-1 antisense-transfected cells.

D. Measurement of E2F-1 Protein Level.

The levels of E2F-1 protein in the T98G and TASA cells after serum stimulation and DEX induction were determined as follows. The cell lines were made quiescent and subsequently stimulated with 10% fetal calf serum for 24 hours with or without DEX. Nuclear protein extracts (200 μg) were loaded onto a 10% acrylamide gel, and a Western blot was performed with an E2F-1-specific polyclonal antibody (UBI, Lake Placid, N.Y.), following the manufacturer's instructions. The results are set forth in Fig. 2 (lane 1, quiescent T98G cells; lane 2, quiescent TASA cells; lane 3, T98G cells+FCS; lane 4, T98G cells+FCS+DEX; lane 5, TASA cells+FCS; lane 6, TASA cells+FCS+DEX). Inhibition of E2F-1 protein expression upon serum stimulation of TASA cells corresponded with induction of E2F-1 antisense transcripts.

Example 2

Altered Cell Cycle Kinetics in Human Glioblastoma Cells Conditionally Expressing E2F Antisense Transcripts

The effects on the cell cycle of down-regulating E2F-1 expression in T98G human glioblastoma cells was investigated as follows. Confluent monolayers of T98G and TASA cells were starved for 4 days in medium containing 0.1% FCS. Twelve hours before stimulation with 10% FCS, either 1 μM DEX or saline solution was added to the cells. Cells were trypsonized, and 5×10^5 cells were plated in 25-cm^2 flasks in medium containing 10% FCS with or without DEX. This experiment was repeated three times with similar results. Cellular DNA content was assayed by the propoidium iodide staining method. Briefly, cells were fixed with 80% ethanol for 15 min. at 4°C, washed and resuspended (10^6/ml) in 1 ml phosphate buffered saline containing 0.1% NP-40 and 1 μg/ml DNase-free pancreatic RNase. Cells were then incubated for 10 min. at room temperature. Five μg of propidium iodide (Sigma Chemical Co., St. Louis, MO) were then added to each sample. Cytofluorimetric analysis was carried in a Coulter Profile II instrument with the software program MULTICYCLE (Phoenix Flow Systems). The results are shown in Fig. 3A-C. Fig. 3A is a plot of the percentage of cells (T98G; TASA) in the S phase versus time. The percentage of cells in the G_2 phase is given in Fig. 3B (T98G) and Fig. 3C (TASA).

Under the conditions of the foregoing cell cycle assay, the effects on the cell cycle of down-regulating E2F-1 expression should be maximized. It was noted that E2F-1 protein levels peaked during the G_1-S transition of the cell cycle. Although steroid induction

somewhat perturbed cell cycle progression of T98G cells, the cytofluorometric assay showed that such treatment resulted in a marked delay in the completion of S-phase in the TASA cell line. In fact, 22 hours after serum stimulation in the presence of DEX, TASA cells were still in the S-phase, while almost 40% of the unstimulated cells had completed DNA synthesis. Forty percent of T98G cells conditionally expressing E2F-1 antisense transcripts were detected in G_2 twenty-eight hours after serum stimulation, a time point at which all control lines were already traversing the second cell cycle (data not shown). This experiment provides direct evident that E2F-1 is, at least partially, required in the S-phase of the cell cycle.

Example 3

Transactivation of Cell Cycle Genes By E2F-1

To determine whether the functional requirement of E2F-1 in the S-phase of the cell cycle correlated with the activation of genes involved in cell cycle progression, the ability of the E2F-1 protein to bind and transactivate the promotors of human DNA polymerase-α, cdc2, c-myb and cyclin D1 was investigated. These genes contain potential E2F sites in their promotors within the first 150 nucleotides preceding the transcriptional start site.

A. Band Shift Assay

The interaction of the E2F-1 protein with the promotors containing the putative E2F binding sites was assessed by the following band shift assay. Double-stranded oligonucleotides corresponding to the promotor genes containing putative EF2 binding sites were obtained after annealing of the complementary single strands. The double-stranded oligonucleotides were

labeled by polynucleotide kinase and [γ^{32}P] ATP. The oligonucleotides used in the assay were as follows:

DNA pol-α:

5'-TCATCACAGGGCGCCAAACGCGCGTCCGC-3' ← (SEQ ID NO:3)
3'-GTAGTGTCCCGCGGTTTGCGCGCAGGCGC-5'

cdc2:

5'-TTTCCTCTTTCTTTCGCGCTCTAGCCACCCG-3'←(SEQ ID NO:4)
3'-AAGGAGAAAGAAAGCGCGAGATCGGTGGGCC-5'

cyclin D1:

5'-GCTGCTCCCGGCGTTTGGCGCCCGCGCC-3' ←(SEQ ID NO:5)
3'-GACGAGGGCCGCAAACCGCGGGCGCGGG-5'

c-myb:

5'-GGGCGCCAGATTTGGCGGGAGGGGGAGT-3' ←(SEQ ID NO:6)
3'-CCGCGGTCTAAACCGCCCTCCCCCTCAC-5'

E2:

5'-GATCCACTAGTTTCGCGCGCTTTCTA-3' ←(SEQ ID NO:7)
3'-TAGGTGATCAAAGCGCGCGAAAGATC-5'

After gel purification, probes were ethanol precipitated and 5 x 10^4 cpm of probe were mixed with 500 ng of a glutathione S-transferase (GST)-E2F recombinant protein (Kaelin et al., Cell 70: 351-364 (1992)) or 500 ng GST and run on a 5% polyacrylamide gel containing 0.5X Tris borate-EDTA buffer. The results of the assay are shown in Fig. 4 (lanes 1 and 2, E2 promotor; lines 3 and 4, DNA polymerase-α promotor; lanes 5 and 6, cdc2 promotor; lines 7 and 8, cyclin D1 promotor; lines 9 and 10, c-myb promotor). Odd and

even numbers in Fig. 4 correspond to +GST and +E2F, respectively.

It may be seen in the band-shift assay of the bacterially produced GST-E2F-1 fusion protein and the various promotors, the double stranded oligonucleotides encompassing E2F sites of the DNA polymerase-α, cyclin D1 and c-myb promotors all bound E2F-1 more efficiently than the viral E2 promotor, whereas GST alone did not bind (Fig. 4). These results indicate that recombinant E2F-1 protein binds to and transactivates human promotors of genes, which have previously been established to be important in the control of cellular proliferation. Surprisingly, however, the oligonucleotide representative of the cdc2 promotor, encompassing the putative high affinity E2F site, was not shifted by the E2F-1 protein.

Example 4

E2F-1 Transactivation of Human Genes With Various Human Promotors

E2F-1 and various human promotors linked to a chloramphenicol acetyltransferase (CAT) reporter gene were coexpressed in different human cell lines to ascertain whether E2F-1 is capable of directly transactivating human genes.

A. Preparation of Promotor-CAT Constructs.

The c-myb promotor construct P1-CAT and the cdc2 promotor construct Pst1 CAT have been described by Nicolaides et al., Mol. Cell. Biol. 11, 6166-6176, (1991) and Ku et al., J. Biol. Chem. 268, 2255-2259 (1993).

The DNA polymerase-α promotor construct DPA-CAT was obtained as follows. A polymerase chain reaction

(PCR) amplification product of the region from -853 to +80 of the DNA polymerase-α promotor was obtained with primers derived from the published sequence and cloned into the PCR1000 cloning vector (InVitrogen). The region from -141 to +80 was then linked to the vector pUC-CAT.

The cyclin D1 promotor construct D1-CAT was prepared as follows. A 2.4 Kb of the human cyclin D1 5' flanking sequence (Slansky et al., Mol. Cell Biol. 13:1610-1618, 1993) extending to nucleotide -1095 starting from the cap site was subcloned in the pUC-7 vector and sequenced. The D1-CAT construct was obtained by subcloning an XhoI-PvuII portion of the cyclin D1 promotor extending from nucleotide -314 to +153 and containing the E2F binding site into the pUC-CAT vector.

The CMV-E2F construct has been described by Kaelin et al., Cell 70: 351-364, 1992.

B. Transient Transfections and CAT Analysis.

Promotor-CAT constructs were cotransfected into T98G cells with the CMV-E2F-1 expression vector, according to the calcium phosphate precipitation method (Nicolaides et al., J. Biol. Chem. 267: 19665-19675, 1992). Briefly, 2 μg of the reporter vector and 10 μg of the CMV-E2F or the empty CMV vector were transfected along with 1 μg of pSV-β-Gal used as an internal control to monitor transfection efficiency. After 48 hours, cells were collected and lysates obtained by freeze-thawing were normalized by a β-galactosidase assay as described by the manufacturer (Promega). Cell lysates were subjected to CAT assay as previously described (Nicolaides et al., J. Biol. Chem. 267: 19665-19675, 1992).

The CAT assay results are shown in Fig. 5A. Transactivation of the different human promotor constructs by E2F-1 is given with respect to the basal CAT activity. The activity of the cdc2 promotor gene was slightly down-regulated by the expression of E2F-1. The promotor constructs used in the study are shown in Fig. 5B wherein the darkened boxes represent the CAT gene and the open boxes indicate the nucleotide positions of the different E2F-1 sites. The degree of transactivation by E2F-1 varied from 4- to 11-fold, depending upon the promotor.

Consistent with the binding assay of Example 3, no transactivation of the cdc2 promotor was observed. Constructs that included more extensive portions of the various promotors did not result in a significant change in CAT activity (data not shown). Identical results were obtained using the SAOS-2 osteosarcoma cell line, which lacks a functional RB protein. In fact, transactivation of the DNA polymerase-α promotor was even higher (~10-fold) in this cell line (data not shown).

Example 5

Transactivation of the c-myb Promotor by E2F-1 After Mutation of the E2F Site.

To ascertain whether the transactivation by E2F-1 was actually dependent on DNA binding, the E2F-1 binding site located at position -59 in the c-myb promotor was mutated.

The construct P1-mut CAT, comprising a PstI-NcoI c-myb promotor fragment linked to the CAT vector, was obtained as follows. A 338-bp PCR product was obtained by priming the E1 myb plasmid (Nicolaides et al., Mol. Cell. Biol. 11: 6166-6176, 1991) with a 5' primer containing a PstI restriction site upstream of the E2F

binding site mutated to TTaCCtGG (TGCGCACTGC AGGGGCGCCA GATTACCTGG GAGG, SEQ ID NO:8) and a 3' primer downstream of an endogenous NcoI (CATCCTCGTC ACTGCTATA, SEQ ID NO:9). The PCR product was digested with NcoI blunt-ended with Klenow, digested with PstI and ligated into the pUC-CAT vector which had been SalI-restricted, blunt-ended with Klenow and PstI-restricted. Sequencing confirmed the induction of the mutation in the E2F-1 site.

P1-CAT (wild type c-myb promotor) or P1-mut CAT (mutant c-myb promotor) was used to transfect T98G cells with or without cotransfection with CMV-E2F. The results of a representative experiment showing CAT activity are shown in Fig. 6A. The isolated spots from the Fig. 6A experiment were excised and ^{14}C radioactivity was measured in a scintillation counter. The results are shown in Fig. 6B.

In repeated experiments, the activity of the mutant promotor plus E2F-1 was approximately 30% of that of the wild-type promotor plus E2F-1. The residual activity was probably due to putative low affinity E2F binding sites scattered between position -40 and +65 in the c-myb promotor. A gel shift assay with the wild type and mutant PstI-NcoI c-myb promotor fragments and the E2F-1 protein revealed that E2F-1 was still able to interact with the mutant promotor (data not shown). Alternatively, the introduced mutation might not have been sufficient to abolish E2F-1 binding.

The present invention may be embodied in other specific forms without departing from the spirit or essential attributes thereof and, accordingly, reference should be made to the appended claims, rather than to the foregoing specification, as indicating the scope of the invention.

All references cited herein with respect to synthetic, preparative and analytical procedures are incorporated herein by reference.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Calabretta, Bruno

(ii) TITLE OF INVENTION: INHIBITION OF CELL PROLIFERATION BY E2F-1 ANTISENSE

(iii) NUMBER OF SEQUENCES: 9

(iv) CORRESPONDENCE ADDRESS:

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(D) STATE: PA

(E) COUNTRY: USA

(F) ZIP: 19103

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk

(B) COMPUTER: IBM PC compatible

(C) OPERATING SYSTEM: PC-DOS/MS-DOS

(D) SOFTWARE: WordPerfect 5.1

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:

(B) FILING DATE:

(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER: US 08/208,575

(B) FILING DATE: 08-MAR-1994

(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: DeLuca, Mark

(B) REGISTRATION NUMBER: 33,229

(C) REFERENCE/DOCKET NUMBER: TJU-1534

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: (215) 568-3100

(B) TELEFAX: (215) 568-3439

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 Nucleotides
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single stranded
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GTGTCGTCGA CCTGAACTG 19

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 Nucleotides
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single stranded
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

TCCAAGCCCT GTCAGAAATC 20

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 58 Nucleotides
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single stranded
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

TCATCACAGG GCGCCAAACG CGCGTCCGCG TAGTGTCCCG CGGTTTGCGC GCAGGCGC 58

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 62 Nucleotides
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single stranded
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

TTTCCTCTTT CTTTCGCGCT CTAGCCACCC GAAGGAGAAA GAAAGCGCGA GATCGGTGGG 60

CC 62

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 56 Nucleotides
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single stranded
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GCTGCTCCCG GCGTTTGGCG CCCGCGCCGA CGAGGGCCGC AAACCGCGGG CGCGGG 56

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 56 Nucleotides
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single stranded
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GGGCGCCAGA TTTGGCGGGA GGGGGAGTCC GCGGTCTAAA CCGCCCTCCC CCTCAC 56

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 52 Nucleotides
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single stranded
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GATCCACTAG TTTCGCGCGC TTTCTATAGG TGATCAAAGC GCGCGAAAGA TC 52

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 34 Nucleotides
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single stranded
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

TGCGCACTGC AGGGGCGCCA GATTACCTGG GAGG 34

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 Nucleotides
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single stranded
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

CATCCTCGTC ACTGCTATA 19

CLAIMS

1. A pharmaceutical composition comprising a pharmaceutically acceptable carrier and an antisense oligonucleotide specific for E2F-1.

2. A composition according to claim 1 wherein the oligonucleotide is an alkylphosphonate oligonucleoside or phosphorothioate oligonucleotide.

3. A composition according to claim 1 wherein the oligonucleotide is an oligodeoxynucleotide.

4. A composition according to claim 1 wherein the oligonucleotide is capable of forming a stable duplex with a portion of an E2F-1 mRNA transcript.

5. A composition according to claim 4 wherein the oligonucleotide is capable of forming a stable duplex with a portion of an E2F-1 mRNA transcript lying within about 50 nucleotides of the translation initiation codon.

6. A composition according to claim 4 wherein the oligonucleotide comprises from a 12-mer to a 50-mer.

7. A composition according to claim 6 wherein the oligonucleotide comprises from a 15-mer to a 40-mer.

8. An antisense oligonucleotide specific for E2F-1 having a length of from about 8 to about 50 nucleotides.

9. An oligonucleotide according to claim 8 which is an alkylphosphonate oligonucleoside or phosphorothioate oligonucleotide.

10. An oligonucleotide according to claim 8 which is an oligodeoxynucleotide.

11. An oligonucleotide according to claim 8 which is capable of forming a stable duplex with a portion of an E2F-1 mRNA transcript.

12. An oligonucleotide according to claim 11 which is capable of forming a stable duplex with a portion of an E2F-1 mRNA transcript lying within about 50 nucleotides of the translation initiation codon.

13. An oligonucleotide according to claim 12 having a length of from about 15 to about 40 nucleotides.

14. An oligonucleotide according to claim 13 having a length of from about 18 to about 30 nucleotides.

15. A method for inhibiting cell proliferation comprising contacting cells with a proliferation-inhibiting effective amount of an antisense oligonucleotide specific for E2F-1.

16. A method according to claim 15 wherein the oligonucleotide is an alkylphosphonate oligonucleoside or phosphorothioate oligonucleotide.

17. A method according to claim 15 wherein the oligonucleotide is an oligodeoxynucleotide.

18. A method according to claim 15 wherein the oligonucleotide is capable of forming a stable duplex with a portion of an E2F-1 mRNA transcript.

19. A method according to claim 18 wherein the cells comprise cancer cells.

20. A method according to claim 19 wherein the cells are characterized by inactivation of retinoblastoma tumor suppressor gene expression.

21. A method according to claim 20 wherein the cells are cancer cells selected from the group consisting of retinoblastoma, glioblastoma, small cell lung cancer, osteosarcoma, bladder cancer, prostate cancer and breast cancer.

22. An artificially-constructed gene comprising a promotor segment and a segment containing an E2F-1 DNA in inverted orientation such that transcription of said artificially-constructed gene produces RNA complementary to at least a portion of an mRNA transcript of the E2F-1 gene.

23. A method for inhibiting the proliferation of neoplastic cells comprising introducing into such cells an artificially-constructed gene which, upon transcription in said cells, produces RNA complementary to an mRNA transcript of the E2F-1 gene.

24. A method according to claim 23 wherein the artificially-constructed gene is introduced into said cells by transfection, by a transducing viral vector or by microinjection.

25. A method of cancer treatment comprising administering to an individual in need of such treatment an effective amount of an antisense oligonucleotide specific for E2F-1.

26. A method according to claim 25 wherein the cancer is characterized by inactivation of retinoblastoma tumor suppressor gene expression.

27. A method according to claim 26 wherein the cancer is selected from the group consisting of retinoblastoma, glioblastoma, small cell lung cancer, osteosarcoma, bladder cancer, prostate cancer and breast cancer.

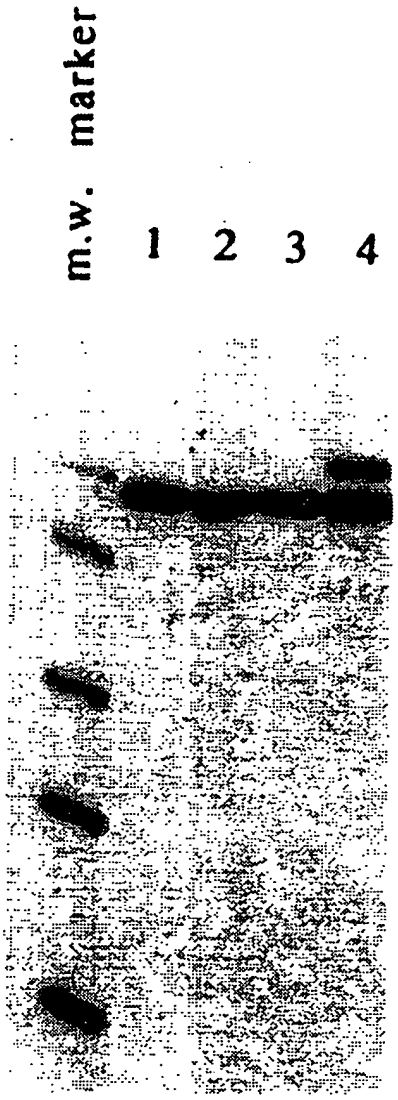

m.w. marker
1 2 3 4
E2F-1 antisense (880bp)
γ-actin (800bp)

FIG. 1

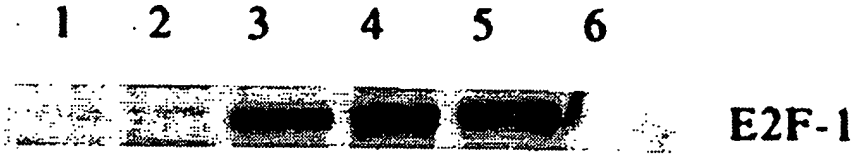

1 2 3 4 5 6
E2F-1

FIG. 2

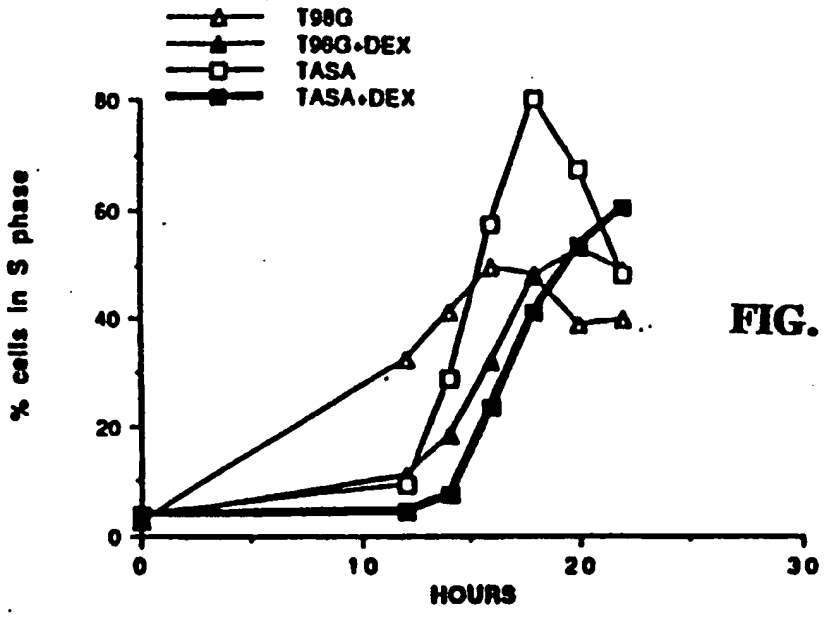


FIG. 3A

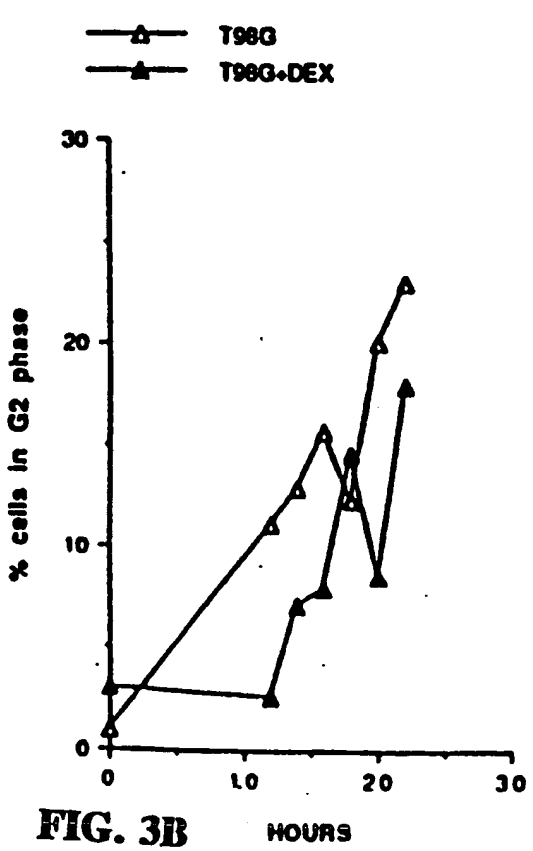


FIG. 3B

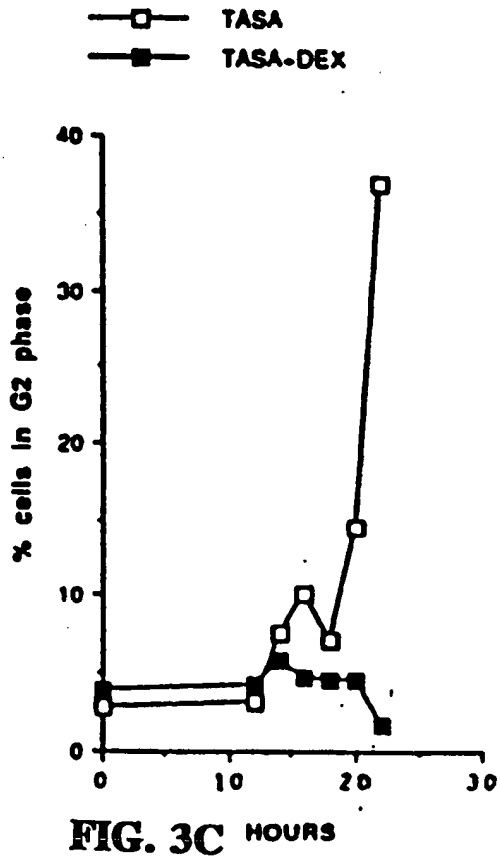


FIG. 3C

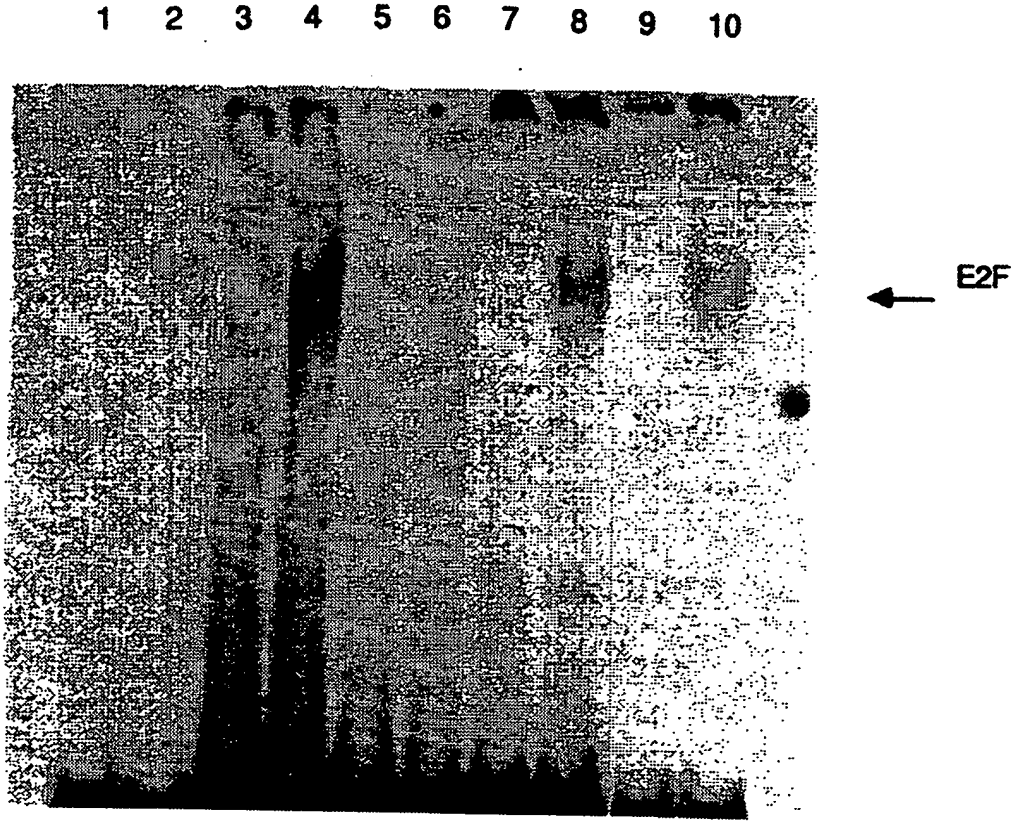


FIG. 4

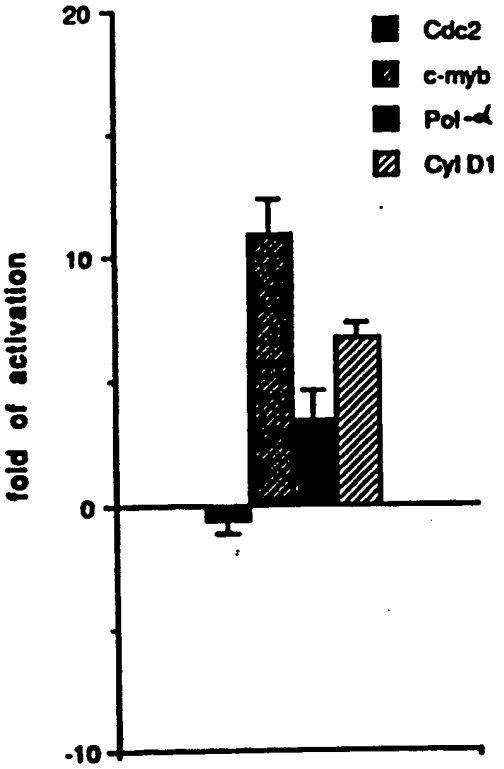

20
10
0
-10
fold of activation
Cdc2
c-myb
Pol-α
Cyl D1

FIG. 5A

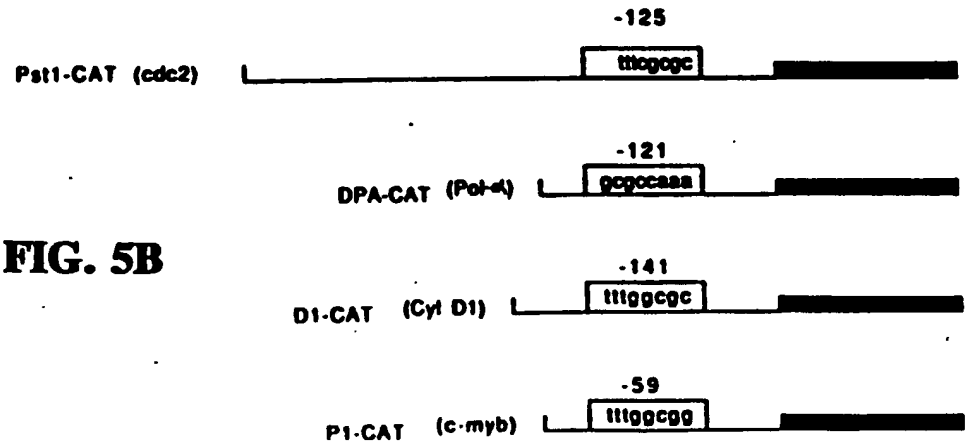

-125
Pst1-CAT (cdc2)
tttgcgc
-121
DPA-CAT (Pol-α)
gcgccaaa
-141
D1-CAT (Cyl D1)
tttggcgc
-59
P1-CAT (c-myb)
tttggcgg

FIG. 5B

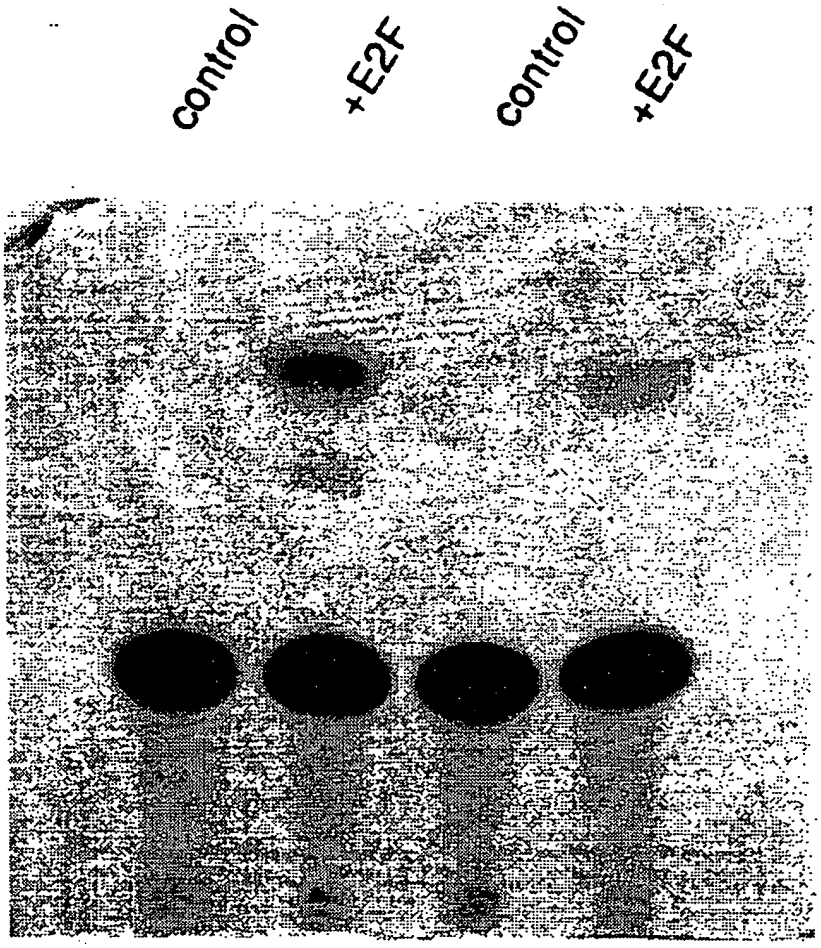
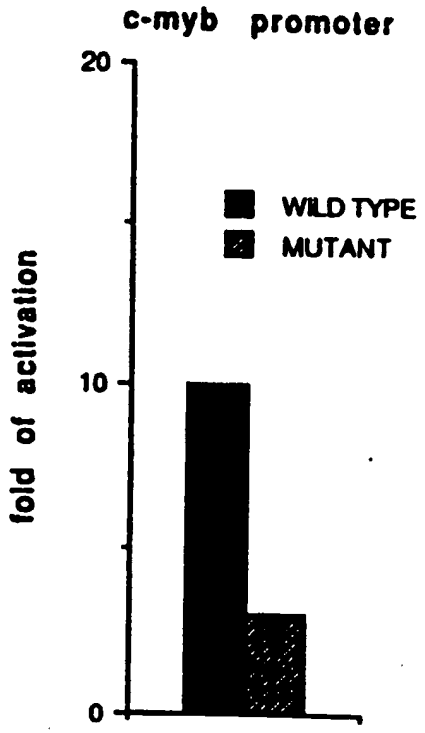


FIG. 6A

FIG. 6B



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/02917

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A61K 48/00, 31/70, 31/74; C12N 15/00, 15/85; C07H 21/00, 21/04
US CL :514/44; 435/172.3; 536/24.5
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/44; 435/172.3; 536/24.5

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, Derwent World Patent Index, BIOSIS
search terms: E2F, cell, cancer?, tumor?, carcino?, neoplas?

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Antiviral Chemistry and Chemotherapy, Volume 2, No. 4, issued 1991, W. James, "Towards Gene-Inhibition Therapy: A Review of Progress and Prospects in the Field of Antiviral Antisense Nucleic Acids and Ribozymes", pages 191-214, see entire document.	1-27
Y	Cell, Volume 70, issued 24 July 1992, W. Kaelin et al., "Expression Cloning of a cDNA Encoding a Retinoblastoma-Binding Protein with E2F-Like Properties", pages 351-364, see entire document.	1-27

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 01 JUNE 1995	Date of mailing of the international search report 08 JUN 1995
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer CHARLES C. P. RORIES Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/02917

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Proceedings of the National Academy of Sciences, Volume 89, issued December 1992, M. Ratajczak et al., "In vivo treatment of Human Leukemia in a scid Mouse Model with c-myb Antisense Oligonucleotides", pages 11823-11827, see entire document.	1-27
Y	Nucleic Acids Research, Volume 20, No. 22, issued 1992, M. Arroyo et al., "Retinoblastoma-Repression of E2F-Dependent Transcription Depends on the Ability of the Retinoblastoma Protein to Interact with E2F and is Abrogated by the Adenovirus E1A Oncoprotein", pages 5947-5954, see entire document.	1-27
Y	Proceedings of the National Academy of Sciences, Volume 86, issued May 1989, S. Hiebert et al., "E1A-Dependent Trans-Activation of the Human MYC Promoter is Mediated by the E2F Factor", pages 3594-3598, see entire document.	1-27
A	Chemical Reviews, Volume 90, No, 4, issued June 1990, E, Uhlmann et al., "Antisense Oligonucleotides: A New Therapeutic Principle", pages 543-584, see entire document.	1-27
A	Science News, Volume 139, issued 16 February 1991, R. Weiss, "Upping the Antisense Ante, Scientists Bet on Profits from Reverse Genetics", pages 108-109, see entire document.	1-27
A	Science, Volume 261, issued 20 August 1993, C. Stein et al., "Antisense Oligonucleotides as Therapeutic Agents - Is the Bullet Really Magical", pages 1004-1012, see entire document.	1-27
A	Cancer Gene Therapy, Volume 1, No. 1, issued March 1994, B. Tseng et al., "Antisense Oligonucleotide Technology in the Development of Cancer Therapeutics", pages 65-71, see entire document.	1-27